A Low Molecular Weight DNA Polymerase from Ovaries of the Frog Xenopus laevis

DNA POLYMERASE-β (OVARIAN)†

(Received for publication, July 19, 1977)

HANS JOENJE‡ and ROBERT M. BENBOW

From the Mergenthaler Laboratory for Biology, The Johns Hopkins University, Baltimore, Maryland 21218

A low molecular weight DNA polymerase (DNA nucleotidytransferase; EC 2.7.7.7) has been purified 265,000-fold from ovaries of the frog Xenopus laevis. On polyacrylamide gels run under denaturing conditions the purified preparation exhibited one major band of approximately 45,000 molecular weight. The most purified fraction incorporated 380 µm of dTMP/min/mg of protein at 26°C. No endonuclease, exodeoxyribonuclease, ribonuclease, or ribonuclease H activity was detected in the most purified fraction.

The purified activity exhibited a Stokes' radius of 29.5 ± 1 Å, as determined by gel filtration on Sephadex G-100, and a sedimentation coefficient of 3.5 S, determined by zone sedimentation in sucrose gradients. From these parameters and assuming a partial specific volume of 0.71 cm³/g a native molecular weight of 45,500 was calculated using the Siegel-Monty relationship.

The purified activity exhibited an optimum at pH 8.7 to 9.1 and was stimulated by 0.1 M NaCl, KCl, or CsCl. With poly(A)·oligo(dT) templates, the purified activity was absolutely dependent upon Mn²⁺ ions and was inhibited by Mg²⁺ ions. In contrast, activity with poly(dA)·oligo(dT) and activated DNA templates utilized either Mn²⁺ or Mg²⁺ as cofactor. The purified DNA polymerase was inactivated by preincubation with 30 µM p-chloromercuribenzoate, but not by N-ethylmaleimide at concentrations up to 10 mM.

Most of the properties of the low molecular weight DNA polymerase purified from ovaries of X. laevis are consistent with the enzyme being a polymerase of the β type.

In eukaryotic organisms at least three separate classes of DNA polymerase activity have been described. These have recently been designated DNA polymerases-α, -β, and -γ (1). DNA polymerases of the β type were defined as low molecular weight enzymes which function optimally at high pH in the presence of high concentrations of KCl or NaCl and are resistant to sulfhydryl reagents. β-Polymerases were also defined as being able to efficiently utilize the hybrid initiator template poly(A)·oligo(dT).

Low molecular weight DNA polymerases (32,000 to 50,000) have been (partially) purified from a variety of eukaryotic cells: calf thymus (2), human KB cells (3), rat liver (4), rat ascites hepatoma cells (5), rabbit bone marrow (6), Novikoff hepatoma (7), and chick embryo (8). In addition, Brun et al. (9) have described two low molecular weight DNA polymerases present at similar levels in the chick embryo with molecular weights of 27,000 and 50,000. The function of these low molecular weight DNA polymerases in vivo is unknown, although they have been postulated to be involved in DNA repair (10).

Benbow et al. (11) have recently developed a cell free DNA replication system based on cytoplasm prepared from unfertilized eggs of the frog, Xenopus laevis. The cytoplasm was fractionated by DEAE-cellulose column chromatography and seven fractions were reconstituted to form a multi-enzyme system which was able to replicate supercoiled DNA molecules. One of the active fractions (Fraction IV) contained two DNA polymerase activities: one was an enzyme of high molecular weight which was unable to use poly(A)·oligo(dT) (i.e. DNA polymerase X-II (now called α (ovarian) of Benbow et al. (12)); and one was of low molecular weight which efficiently used poly(A)·oligo(dT). The purification and some properties of the low molecular weight DNA polymerase are described in this paper.

EXPERIMENTAL PROCEDURES

Frogs—Adult Xenopus laevis females were obtained from the South African Snake Farm (P. O. Box 6, Fish Hoek, Cape Province, South Africa) and were maintained according to Gurdon (13). The average weight of a single ovary varied from 6 to 20 g depending on the season, with the highest yields in August and September. Buffers—Buffer A contained 25 mM Tris-HCl (pH 7.8, except

† The enzymes are indicated α (ovarian) or β (ovarian) to indicate the source from which the enzyme was isolated (oocytes) to distinguish it from similar activities isolated from eggs or embryos; preliminary evidence suggests that Xenopus laevis DNA polymerases are modified during embryogenesis (R. Lennox, H. Joenje, and R. M. Benbow, unpublished observations).
DNA Polymerase-β (Ovarian) of Xenopus laevis

2641

when stated otherwise), 0.2 mM dithioerythritol, 0.4 mM EDTA, 350 mM (v/v) glycerol, and indicated concentrations of KCl. Buffer B contained 0.2 mM dithioerythritol, 0.4 mM EDTA, 3 mM MgCl₂, 250 mM glycerol, and the indicated concentrations of Tris-HCl (pH 8.0). Buffer C contained 0.2 mM dithioerythritol, 0.4 mM EDTA, 0.2 mM KCl, 250 mM (v/v) glycerol, and the indicated concentration of potassium phosphate (pH 7.6). All pH values were measured at room temperature.

Column Chromatography and Gel Filtration Media—DEAE-cellulose (type DE52 from Whatman Biochemicals Ltd., Maidstone, England) was equilibrated with Buffer B from which glycerol and dithioerythritol had been omitted, washed with three changes of Buffer C, and complete starting buffer, and used for chromatography within 3 days. Phosphocellulose (cellulose phosphate type P11 from Whatman) was pretreated (14) and equilibrated with Buffer A as described above for DEAE-cellulose. Hydroxylapatite (Bio-Gel HT from Bio-Rad Laboratories, Richmond, Calif.) was equilibrated with Buffer A plus 0.2 M KCl and bovine serum albumin (200 μg/ml) as described by the manufacturer and stored in the presence of 0.05% sodium azide at -20°F until use. Columns of 2.5-cm diameter were pored and washed before use for 2 days with the same buffer at an operating pressure of 60 cm, giving a flow rate of 30 ml/h. Single-stranded DNA cellulose (0.5 mg of DNA/ml settled volume) was prepared from cellulose (Cellex N-1 from Bio-Rad Laboratories) and heat-denatured calf thymus DNA (type I from Sigma Chemical Co., Saint Louis, Missouri) as described by Alberts and Herrick (15).

Reconstruction of Column Gradients—Twenty-five micrograms aliquots of column fractions were dialyzed 40-fold with H₂O and the conductivity was measured with a conductivity meter (type CDM 3 from Philadelphia, Pennsylvania). DNA Polymerase Assays—Two different assays were used. Assay 1 measured low molecular weight DNA polymerase activity per ml in a total volume of 0.5 ml; incubation was for 60 min at 30°C, after which 10 mM EDTA was added. DNA was extracted with phenol and examined by electron microscopy (21). The sensitivity of the assay was such that a carrier DNA solution (0.5 mg/ml of calf thymus DNA) was heated at 95°C for 5 min, chilled in ice, and mixed with 0.6 ml of 10% trichloroacetic acid. After standing at 0°C for 15 min, the tubes were centrifuged for 23 h at 40,000 rpm, 3°C. Gradients containing the polymerase activity were fractionated, and 10 mM EDTA was added. DNA was extracted with phenol and ethanol and examined by electron microscopy (21). The sensitivity of the assay was such that a concentration of 10 mg/ml of pancreatic deoxyribonuclease I (Sigma Chemical Co.; 1700 Kunitz units/mg as specified by Sigma) converted virtually all of the supercoiled molecules into relaxed circles and lines.

Exonuclease activity was measured on native and heat-denatured "H-labeled calf thymus DNA (all four bases were labeled) in 0.1-M reaction mixtures containing 50 mM Tris-HCl (pH 8.0), 5 mM MgCl₂, 2.5 mM β-mercaptoethanol, 250 μg/ml of bovine serum albumin, and [3H]labeled DNA (16 μM, 100 cpm/pmol) (20), and 22 units of DNA polymerase-β (ovarian). Exonuclease activity with native DNA was also measured in the presence of dATP, dCTP, dGTP, and dTTP (75 μM each). After 50 and 60 min incubations at 30°C, 0.5 ml of a denatured DNA solution (see legend to Fig. 1A) was added to each reaction mixture. The tubes were centrifuged for 20 min at 3000 × g and 0.5-ml samples of the supernatants were counted with 8 ml of water-miscible scintillation fluid (solution "So46" of Research Products International). In this assay, using nonadenated DNA, 5 units of Escherichia coli exonuclease III (Miles Laboratories) digested 60% of the total radioactivity in 30 min; 1.8 units of E. coli DNA polymerase I (Grade I of

M. R. Krause, unpublished observations.

R. H. Reeder, personal communication.
DNA Polymerase-β (Ovarian) of Xenopus laevis

Table I

Purification of DNA polymerase-β (ovarian) from Xenopus laevis ovaries

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume (ml)</th>
<th>Total protein (mg)</th>
<th>Total units dTMP/μl</th>
<th>Specific activity (units/mg)</th>
<th>Purification 50% Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Crude extract</td>
<td>8,400</td>
<td>231,000</td>
<td>336,000</td>
<td>1.45</td>
<td>1</td>
</tr>
<tr>
<td>II. Low speed supernatant</td>
<td>7,700</td>
<td>58,500</td>
<td>268,000</td>
<td>4.6</td>
<td>3.2</td>
</tr>
<tr>
<td>III. High speed supernatant</td>
<td>7,520</td>
<td>54,900</td>
<td>282,000</td>
<td>4.6</td>
<td>3.2</td>
</tr>
<tr>
<td>IV. DEAE-cellulose</td>
<td>4,200</td>
<td>7,800</td>
<td>19,100</td>
<td>0.58</td>
<td>100</td>
</tr>
<tr>
<td>V. Phosphocellulose</td>
<td>356</td>
<td>107</td>
<td>72,600</td>
<td>3.6</td>
<td>80</td>
</tr>
<tr>
<td>VI. Hydroxyapatite</td>
<td>29</td>
<td>9.1</td>
<td>93,000</td>
<td>1.7</td>
<td>22</td>
</tr>
<tr>
<td>VII. Sephadex G-100</td>
<td>50</td>
<td>0.185</td>
<td>35,500</td>
<td>192,000</td>
<td>172</td>
</tr>
<tr>
<td>VIII. DNA-cellulose</td>
<td>5.7</td>
<td>0.064</td>
<td>24,500</td>
<td>384,000</td>
<td>265,000</td>
</tr>
</tbody>
</table>

a Protein in Fractions I through VI was determined using the procedure of Lowry et al. (16); in Fractions VII and VIII protein was estimated from the absorbance at 280 nm, assuming that 1 A280 unit corresponds to 1 mg of protein.

b Assays were incubated for 30 min at 26° and were linear with enzyme concentration, except for Fraction IV. All assays contained 0.25 M KCl to avoid measuring activity from a high molecular weight DNA polymerase responding to assay 2 at low salt concentration, since at this salt concentration, the low molecular weight DNA polymerase showed 50% of its maximal activity, the values found were multiplied by 2 to give the values presented.
c Activity was measured after dialyzing a 1 ml aliquot against Buffer A plus 5 mM KCl for 3 h to lower the Mg2+ concentration, which strongly inhibits assay 2 (Fig. 6A). 1-ml samples were assayed. No interference with inhibitors occurred in these assays, as judged by the fact that no inhibition was observed when a known amount of Fraction VII enzyme was mixed with 1-ml aliquots of Fractions I, II, and III.
d Activity in the pooled DEAE-cellulose fractions was severely underestimated because it was (reversibly) inhibited by material that eluted at slightly higher ionic strength and partly overlapped with the DNA polymerase.
e Phosphate concentration in the assay was below 1 mm.

Boehringer Mannheim GmbH digested 7% of the input radioactivity in 30 min. The limit of detection of the exonuclease assay was approximately 1.5 pmol of solubilized nucleotides (0.1% of the input radioactivity). Ribonuclease activity was measured in 0.1 ml reaction mixtures containing 50 mM Tris-HCl (pH 7.6), 5 mM MgCl2, 30 mM NaCl, 250 μg/ml of bovine serum albumin, 3H-labeled 4 S RNA from E. coli (Miles Laboratories; 11 μM; 230 cpm/μmol), and 22 units of low molecular weight DNA polymerase activity. After 30 and 60 min at 30°, acid-soluble radioactivity was determined as described above for the exonuclease assay. The detection limit of this assay was 11 pmol of solubilized ribonucleotides.

Ribonuclease H (22) activity was measured in 0.1 ml reaction mixtures containing 50 mM Tris-HCl (pH 7.6), 100 mM KCl, 1 mM β-mercaptoethanol, 250 μg/ml of bovine serum albumin, 1 mM MgCl2, 10 μM 3H-labeled poly(A) (Schwarz/Mann; 12 cpm/μmol) hybridized (5 min, 45°) with 40 μM poly(d(T)) (P-L Biochemicals), and 22 units of DNA polymerase β (ovarian). Incubation was for 30 and 60 min at 30°. Acid-soluble radioactivity was determined as described above. Five microliters of a ribonuclease H preparation extensively purified from X. laevis ovaries (an amount equivalent to that contained in 1 mg of ovary) digested 60% of the input radioactivity in 30 min. The limit of detection was approximately 4 pmol of solubilized ribonucleotides (0.4% of the input radioactivity).

RESULTS

All manipulations were carried out at 0-5°. The purification (Table I) is based on 985 g of Xenopus laevis ovaries, obtained from 70 adult female frogs. For small scale purifications all steps must be scaled down to ensure stabilization of the enzyme.

Crude Extract—Frogs were decapitated, ovaries removed, and homogenized for 2 min with a Waring Blender at full speed in a solution (8 ml/g of ovary) containing 25 mM Tris base (pH 9.3), 3 mM MgCl2, 0.4 mM KI, 1 mM β-thioglycerol, 25% (v/v) glycerol. A total volume of 8400 ml of dark gray extract was obtained; the pH was approximately 7.1, and most of the black pigment granules. The supernatant was covered with a yellow layer of lipids, most of which was removed by filtration through eight layers of cheese cloth to give 7700 ml of turbid dark brown low speed supernatant (Fraction I).

High Speed Centrifugation—Low speed supernatant was centrifuged at 95,000 x g for 90 min to remove mitochondria, remaining pigment, and lipids. The supernatant was carefully decanted from the loose pellet and filtered through eight layers of cheese cloth to remove the lipid pellets. The slightly turbid reddish yellow high speed supernatant (Fraction III; 7520 ml) was stored at -20°.

DEAE-cellulose Chromatography—600- to 700-ml portions of DEAE-cellulose equilibrated with 50 mM Tris·HCl in Buffer B were aspirated to near dryness on a 3-liter Buchner funnel fitted with a fritted glass filter, mixed with 750-ml portions of Fraction III, and stirred for 90 min. At least 93% of the enzyme activity was adsorbed during this period. Nonadsorbed material was removed by filtration, the cellulose was washed three times with 400 ml of starting buffer, resuspended in 400 ml, and allowed to settle in a 5-cm diameter glass column. The resulting bed (~5 x 32 cm) was eluted with a 2-liter linear Tris·HCl gradient of 0.05 M to 0.4 M in Buffer B at a flow rate of 120 to 160 ml/h, followed by a wash with 500 ml of 0.4 M Tris·HCl in Buffer B, 22.5-ml fractions were collected.

The elution pattern is shown in Fig. 1A. Three peaks of 280 nm absorbing material were eluted at approximately 0.1 M, 0.15 M, and 0.25 to 0.35 M Tris·HCl; the first contained red material, presumably hemoglobin. DNA polymerase activities responding to assay 1 eluted at approximately 0.14 M and 0.24 M Tris·HCl, corresponding to the high molecular weight DNA polymerases X-II (now called α (ovarian)) and X-I (now γ (ovarian)) of Benbow et al. (12). DNA polymerase activity responding to assay 2 eluted at approximately 0.15 M Tris·HCl, coinciding with the second A280 nm peak. This peak was pooled and dialyzed against Buffer A plus 0.18 M KCl (two changes of 9 volumes over a period of 24 h). After pooling, the measured activity dropped dramatically due to the presence of material eluting at approximately 0.2 M Tris·HCl that reversibly inhibited assay 2. This inhibitor was subsequently re-
moved by phosphocellulose chromatography in Step V.) The pooled dialyzed peaks from 10 DEAE-cellulose columns were combined to constitute Fraction IV (420 ml).

**Phosphocellulose Chromatography**—Fraction IV was applied at a flow rate of 150 ml/h to a column (5 x 26 cm) containing 500 ml of phosphocellulose equilibrated with Buffer A plus 0.18 m KCl. After washing with 3 column volumes of starting buffer, the column was eluted with a 2-liter linear KCl gradient of 0.18 to 0.8 m in Buffer A at a flow rate of 150 ml/h; 22.5-ml fractions were collected. As shown in Fig. 2A, polymerase activity eluted at approximately 0.28 m KCl, partly overlapping with the low molecular weight DNA polymerase (responding to assay 2) which eluted at approximately 0.3 m KCl. The latter activity was pooled as indicated in Fig. 1B to give Fraction V (356 ml).

**Hydroxylapatite Chromatography**—Fraction V was pumped at 50 ml/h onto a column (3 x 6 cm) of hydroxylapatite equilibrated with Buffer A plus 0.18 m KCl. After washing with 3 column volumes of Buffer A plus 0.5 m KCl. The column was washed with 120 ml of Buffer A plus 0.5 m KCl followed by 160 ml of 0.02 m potassium phosphate in Buffer C, eluted with a 140-ml linear potassium phosphate gradient of 0.02 to 0.15 m, and washed with 0.25 m potassium phosphate in Buffer C. The flow rate was 50 ml/h and fractions of 4 ml were collected. As shown in Fig. 2A, polymerase activity eluted at approximately 0.1 m potassium phosphate. Since the activity is unstable at low protein concentrations, it is advisable to collect the fractions in 200 µg/ml of bovine serum albumin (final concentration). To be able to measure the specific activity of the enzyme accurately, we did not use this protective treatment, but proceeded quickly instead. The pooled activity (38 ml) was concentrated by pressure dialysis to 2.9 ml (Fraction VI) using a Diaflo ultrafiltration apparatus with a PM 10 filter (Amicon Corp., Lexington, Mass.).

**Gel Filtration through Sephadex G-100**—Fraction VI was applied and eluted as described in the text. 0.5-ml aliquots from each fraction were assayed for DNA polymerase activity (●) with assay 2; incubation was for 20 min at 37°. The A

\[ \text{Absorbance at } 260 \text{ nm} \]

was measured against equilibration buffer. The A

\[ \text{Absorbance at } 260 \text{ nm} \]

of the fractions contained approximately 25% of the total input of DNA absorbance material. The A

\[ \text{Absorbance at } 260 \text{ nm} \]

due to the presence of bovine serum albumin in the fractions has been subtracted from the measured A

\[ \text{Absorbance at } 260 \text{ nm} \]

of the fractions.
applied to a column (2.5 x 48 cm) of Sephadex G-100 equilibrated with Buffer A plus 0.2 M KCl and 200 µg of bovine serum albumin/ml; 3.3-ml fractions were collected. The fractions indicated in Fig. 2B were pooled to give Fraction VII.

Affinity Chromatography on Single-stranded DNA Cellulose — Fraction VII was applied at 20 ml/h to a single-stranded DNA cellulose column (0.9 x 5.5 cm) equilibrated with Buffer A (pH 7.8) plus 0.2 M KCl and 200 µg of bovine serum albumin/ml. The column was washed with 25 ml of this buffer followed by a 30-ml linear KCl gradient of 0.2 to 0.8 M in Buffer A plus 200 µg of bovine serum albumin/ml. The flow rate was 20 ml/h; fractions of 1.1 ml were collected. DNA polymerase activity eluted at approximately 0.4 M KCl, coinciding with a small peak of 280 nm absorbing material. The fractions indicated in Fig. 2C were pooled (5.7 ml) and frozen at -20° in 0.1-ml aliquots containing 4.3 units of DNA polymerase activity per µl. At -20° the activity was stable for at least 3 months when stored in the presence of bovine serum albumin, EDTA, glycerol, and dithioerythritol. Bovine serum albumin can be removed by adsorbing the enzyme to a DNA-cellulose column at 0.2 M KCl (10,000 units/ml of DNA cellulose), washing, and eluting with buffer plus 0.6 M KCl.

Specific Activity — The final preparation was purified 265,000-fold from the crude extract (Table I) to a specific activity of 384,000 units/mg of protein (or 105 mKat/kg according to the nomenclature recently proposed (23)) with a yield of 7.3%. Fraction VIII enzyme was tested for exon- and endoexonuclease activity, ribonuclease, and ribonuclease H activities specified under "Experimental Procedures." One strong band with an RI; value of 1.75, indicating less than 1% contamination by nucleic acids. The amount of protein in 2450 units was measured by the method of Lowry (16), giving a value 3-fold less than the A 280 nm. Based on the Lowry estimate the total amount of protein in Fraction VIII would be 0.21 mg with a specific activity of 1.15 x 106 units/mg instead of the values given in Table I.

Electrophoretic Homogeneity — DNA polymerase-β (ovarian) was concentrated and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described under "Experimental Procedures." One strong band with an Rf value of 0.78 was found in this gel (Fig. 3), although other faint bands may be visible. Since the apparent molecular weight of the major band is very similar to the molecular weight of the DNA polymerase-β (ovarian) activity determined by velocity sedimentation and gel filtration, we believe it is reasonable to assume that this band corresponds to the DNA polymerase-β (ovarian) polypeptide chain even though no direct evidence is given in this paper.

Molecular Weight — The Stokes' radius of Fraction VIII enzyme was determined from the elution position of activity from a calibrated Sephadex G-100 column (17). The Stokes' radius (Fig. 4A) was 29.5 ± 1 A. The sedimentation coefficient was determined by the method of Martin and Ames (18) and found to be 3.5 S (Fig. 4B), based on a sedimentation coefficient of 3.66 S for ovalbumin (25). Assuming a partial specific volume of 0.74 (26), a molecular weight of 45,500 was calculated using the equations of Siegel and Monty (17). Biological Characterization — The low molecular weight DNA polymerase required (a) a polynucleotide template strand, (b) a complementary oligonucleotide initiator, (c) deoxyribonucleoside 5'-triphosphate(s), and (d) a divalent cation. With both poly(A)-oligo(dT) and activated calf thymus DNA templates, the pH optimum was 8.7 to 9.1 (Fig. 5A). Maximal incorporation with poly(A)-oligo(dT), poly(A)-oligo(dT), and activated DNA was found at 26°, 30°, and 37°, respectively (Fig. 5B). These temperature optima parallel (at approximately 30° lower) the relative melting temperatures of these template initiator complexes and suggest that DNA polymerase-β (ovarian) destabilizes double helical structures. Moreover, these data suggest that the temperature optima are more a property of the initiator-template complexes than of the DNA polymerase-β (ovarian) molecules. At temperatures up to 30° incorporation of radioactivity was linear for at least 1 h; at higher temperatures incorporation leveled off at or before 30 min suggesting enzyme lability above 30°.

DNA polymerase activity with poly(A)-oligo(dT) increased as a function of KCl or NaCl concentration up to 0.1 M, then slowly decreased (Fig. 5C). Similar stimulation of activity was found for CsCl (2-fold at 0.1 M); however, approximately 25% inhibition was found for NH₄Cl and LiCl at 0.1 M. Similar monovalent cation dependence was observed when activated
DNA was used as template. Among a variety of divalent cations tested, including Cu++, Mg++, Sr++, Ba++, Mn++, Zn++, Co++, and Cu++, only Mn++ served as a cofactor with poly(A)-oligo(dT). The optimum Mn++ concentration was 0.4 to 0.8 mM (Fig. 6A). Not only did Mg++ fail to support DNA synthesis with poly(A)-oligo(dT), it inhibited the Mn++-dependent reaction (Fig. 6A, inset). Such inhibition was not observed with, for example, Ba++. In contrast to these results, DNA polymerase activity with activated calf thymus DNA utilized either Mn++ or Mg++ as cofactor (Fig. 6B). Similar results were obtained when poly(dA)-oligo(dT) was used as template. In preliminary attempts to determine the $K_m$ for dTTP we used poly(A)-oligo(dT) as template with 0.4 mM Mn++ and varied the concentration of dTTP. However, the reaction velocity increased until approximately 0.4 mM dTTP, then declined sharply at higher concentrations so that no $K_m$ could be derived (Fig. 7A) (see also Ref. 29). When 0.1 mM Mn++ was used, the reaction velocity increased only until 0.1 mM dTTP, then decreased (Fig. 7A). We do not understand the basis of this strange dependence on dTTP concentration, but have noted that apparently normal kinetics can be obtained by using Mn++-dTTP in place of dTTP.

Therefore, to obtain a $K_m$ value for dTTP an equimolar amount of MnCl$_2$ was added simultaneously with the dTTP (Fig. 7B); all assays contained in addition a fixed concentration of MnCl$_2$. Under these conditions normal saturation for Mn++-dTTP occurred and no decline of activity at high concentrations (up to 3.75 mM) was observed. A $K_m$ for Mn++-dTTP of approximately 100 $\mu$M was derived from these data.

Different $K_m$ values were obtained when poly(dA)-oligo(dT) was used as template: in the presence of 0.2 mM Mn++ a normal saturation curve was obtained (Fig. 8), from which a $K_m$ of approximately 10 $\mu$M was derived. In the presence of Mn++, however, a sigmoidal saturation curve was obtained (Fig. 8), in which 50% of maximal polymerization occurred at approximately 375 $\mu$M. When activated calf thymus DNA was used as template, normal saturation curves were obtained for both Mg++ and Mn++ with $K_m$ values of 10 to 15 $\mu$M.

The affinity of the low molecular weight DNA polymerase for three initiator-template complexes was estimated (Table II). When the concentration of the template strand poly(A) was kept constant (at 0.1 mM) and the initiator concentration was increased, similar saturation curves were obtained, although the $K_m$ was 3- to 4-fold higher than when both initiator and template were increased simultaneously. This suggests that the $K_m$ observed primarily reflects increases in the initiator concentration. The $K_m$ for poly(dA)-oligo(dT) was similar to the $K_m$ for poly(A) oligo(dT) (Table III), whereas
Fig. 6. Divalent cation dependence of DNA polymerase-β (ovarian) activity with poly(A)·(dT)$_{12-18}$ (A) or activated DNA (B) templates. A, Incubation mixtures were as described under "Experimental Procedures" (assay 2), except that the concentration of MnCl$_2$ (○) or MgCl$_2$ (□) was varied; incubation was for 30 min at 26°C with 1 unit of DNA polymerase activity (Fraction VIII). The inset shows the effect of the indicated concentrations of MgCl$_2$ in the presence of a fixed concentration of MnCl$_2$ (0.25 mM); 100% residual activity corresponded to incorporation of 680 pmol of [H]-dTMP/h. See Stavrianopoulos et al. (27) for a similar observation and Ref. 28 for the contrasting behavior of DNA polymerase-γ. B, Assays contained in a total volume of 0.1 ml: 50 mM Tris·HCl, pH 8.9, KCl (0.1 M), activated DNA (0.4 mM), dATP, dCTP, and dGTP (0.1 mM each), [H]-labeled dTTP (0.1 mM, 100 cpm/pmol), bovine serum albumin (300 μg/ml), approximately 4 units of Fraction VII enzyme, and various concentrations of either MnCl$_2$ (○) or MgCl$_2$ (□). Incubation was for 30 min at 26°C.

Fig. 7. Effect of dTTP concentration on DNA polymerase activity with poly(dA)·(dT)$_{12-18}$ (A), Assays were as described under "Experimental Procedures" (assay 2), except that the MnCl$_2$ concentration was 0.1 mM (○) or 0.4 mM (□) and that the dTTP concentration was varied. The specific activity of the [H]-labeled dTTP was 75 cpm/pmol; incubation was for 45 min at 30°C with approximately 0.1 unit of DNA polymerase activity. B, As in A, except that assays contained not only a fixed concentration of MnCl$_2$ (0.2 mM) but also variable concentrations equal to the dTTP concentration (see text).

the $K_m$ for activated DNA was considerably higher. However, this probably was due to fewer 3' OH groups/unit length of template.

The utilization of selected initiator-template complexes by the DNA polymerase is shown in Table III. (These template preferences are the basis of differential DNA polymerase assays.) Among the polyribonucleotide templates tested, only poly(A)·oligo(dT)$_{12-18}$ was utilized efficiently, provided that Mn$^{2+}$ was present. Oligo(U)$_{12-21}$ was unable to replace oligo(dT)$_{12-18}$ (0.5 pmol/h). Of the polydeoxyribonucleotides tested, only poly(dA), poly(dC), and poly(dT) were used efficiently. Poly(dA) was efficiently utilized with the initiator oligo(dT), but not with oligo(U) (5 pmol/h). In contrast, poly(dT) was more efficiently utilized with the initiator oligo(A) than with oligo(dA). This was also the only case in which a hybrid template-initiator complex was utilized in the presence of Mg$^{2+}$. Activated DNA was a poor template, at least 10-fold less efficient than poly(A)·oligo(dT).

Preincubation of the low molecular weight DNA polymerase with 5 or 30 μM p-chloromercuribenzoate inhibited incorporation by more than 90% or 98%, respectively. This inhibition could be greater than 90% reversed by the thiol-reducing agents 2-mercaptoethanol (1 mM), dithioerythritol (1 mM), and cysteine (1 mM), but not by cysteine (1 mM) which minimizes the possibility that p-chloromercuribenzoate had inactivated the enzyme by reacting with molecular groups other than —SH. These results suggest that the low molecular weight DNA polymerases do require an SH group for activity.

The effect of potential inhibitors on the low molecular weight DNA polymerase activity was tested in the presence of either poly(A)·oligo(dT) or activated DNA (Table IV). Activity with poly(A)·oligo(dT) was very sensitive to the intercalating dye ethidium bromide and fairly sensitive to chloroquine.
The DNA polymerase described in this paper has most of the properties of a β-polymerase (1): it is low molecular weight, is resistant to high concentrations of monovalent cations, exhibits maximal activity at alkaline pH, and efficiently copies poly(A)-oligo(dT) templates. Unlike most β-polymerases, however, the extreme sensitivity of the X. laevis low molecular weight DNA polymerase to β-chloromercuribenzoate indicates that an —SH group is required for activity; note, however, that the —SH group showed little reactivity toward N-ethylmaleimide. This striking difference between the two sulfhydryl reagents could be due to the location and/or microenvironment of the critical —SH group(s) in the enzyme. Since "typical" β-polymerases have been described even to N-ethylmaleimide (7, see also Ref. 34), we feel, considering all the other similarities, that the low molecular weight, is resistant to high concentrations of monovalent DNA polymerase (12) does not affect the low molecular weight DNA polymerase activity. Single-stranded DNA had only a limited inhibitory effect; this differs from the high molecular weight DNA polymerases from rat liver (33) and from X. laevis DNA polymerase-β-ovarian (12). Activity was not significantly inhibited by araCTP nor by tubercidin, but strongly inhibited by sodium phosphate and even more by sodium pyrophosphate.

### DISCUSSION

The DNA polymerase described in this paper has most of the properties of a β-polymerase (1): it is low molecular weight, is resistant to high concentrations of monovalent cations, exhibits maximal activity at alkaline pH, and efficiently copies poly(A)-oligo(dT) templates. Unlike most β-polymerases, however, the extreme sensitivity of the X. laevis low molecular weight DNA polymerase to β-chloromercuribenzoate indicates that an —SH group is required for activity; note, however, that the —SH group showed little reactivity toward N-ethylmaleimide. This striking difference between the two sulfhydryl reagents could be due to the location and/or microenvironment of the critical —SH group(s) in the enzyme. Since "typical" β-polymerases have been described which show moderate sensitivity to mercurial inhibitors and even to N-ethylmaleimide (7, see also Ref. 34), we feel, considering all the other similarities, that the low molecular weight DNA polymerase purified from X. laevis ovaries is a β-polymerase rather than being a representative of a new class.

Other low molecular weight DNA polymerase have been...
DNA polymerase-β (Ovarian) of Xenopus laevis

described in *X. laevis*. For example, Benbow et al. (12, 20) have identified a low molecular weight DNA polymerase, DNA polymerase X-III (now called β (embryonic)), which appeared at high levels during neurulation and hatching in *X. laevis* embryos; this enzyme also exhibited many of the properties (salt sensitivity, alkaline pH optimum) of the β-polymerases, and we also classify it as a β-polymerase; however, we caution that this does not imply identity with the DNA polymerase-β (ovarian).

Tato et al. (35) separated three DNA polymerase activities from *X. laevis* oocytes by phosphocellulose column chromatography. Their activity eluting at 0.32 M KCl efficiently copied poly(A)-oligo(dT) and probably is identical with DNA polymerase-β (ovarian). However, their activity appeared in the pellet after low speed centrifugation and required solubilization with buffer containing 0.5% Triton X-100. In contrast, RNA polymerase-P (ovarian) (Fig. 5, Table III) do not seem to be compatible with those observed with the purified enzyme in vitro.

Purified DNA polymerase-β (ovarian) has been tested in our cell-free DNA replication system (11): no absolute requirement for the ovarian enzyme could be demonstrated using supercoiled polyoma or pXlr plasmid template DNA molecules in vitro.

Acknowledgments — We thank Drs. E. B. Waygood and N. K. Ahmed for valuable suggestions for the purification procedure and M. Krauss for doing the electron microscope assay of endonuclease activity. We are especially grateful to Ronald Lennox for numerous discussions of the research and to Carol Breaux, Nancy Wang, Eric Nelson, and Sonia White for their comments on the manuscript.

REFERENCES

A low molecular weight DNA polymerase from ovaries of the frog Xenopus laevis. DNA polymerase-beta (ovarian).

H Joenje and R M Benbow


Access the most updated version of this article at [http://www.jbc.org/content/253/8/2640.citation](http://www.jbc.org/content/253/8/2640.citation)

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at [http://www.jbc.org/content/253/8/2640.citation.full.html#ref-list-1](http://www.jbc.org/content/253/8/2640.citation.full.html#ref-list-1)